Characterization and Biosynthesis of Proteoglycans of Corneal Stroma from Rhesus Monkey*

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The proteoglycans of the Rhesus monkey corneal stroma were characterized by analyzing both radiolabeled proteoglycans synthesized by corneas in organ culture and native corneal proteoglycans obtained by large scale preparations. The analyses indicate that the proteoglycans synthesized in organ culture were similar to, if not identical with, their counterparts in the stroma although they are synthesized in different proportions in vitro than they accumulate in vivo.

The corneal stroma contains two proteoglycans. The chondroitin-dermatan sulfate proteoglycan consists of ~70% protein and has a M_2 = ~100,000 to 150,000. It contains one chondroitin-dermatan sulfate side chain of M_2 = ~55,000. The keratan sulfate proteoglycan consists of ~74% protein and has a M_2 = ~40,000 to 70,000. It contains one or two keratan sulfate side chains with M_2 = ~7,000 each. Radiolabeling indicates that both proteoglycans contain glycoprotein-type oligosaccharides as part of their structure.

The adult cornea contains three different cellular layers; the epithelium, the stroma, and the endothelium. The stroma constitutes the thickest portion and contains fibroblastic cells called stromocytes which are embryologically derived from the neural crest. The specialized extracellular matrix of the stroma consists of highly ordered orthogonal lamellae composed of type I collagen fibrils and of proteoglycans which contain keratan sulfate and chondroitin sulfate. The organization of the collagen and proteoglycans in the stroma as well as the macromolecular characteristics of the proteoglycans are thought to be responsible for the structure and transparency of the tissue.

Although the characteristics (6-10) and biosynthesis (11-17) of the glycosaminoglycans of corneal tissues have been studied extensively, Jess is known about the properties of the intact proteoglycans, the source of the glycosaminoglycans. Antonopoulos et al. (3) and Axelsson and Heinegård (4) showed that over 85% of the hexosamine content of the stroma was extractable with 4 M guanidine hydrochloride. Subsequently, the proteoglycans in the extract were purified and partially characterized using cesium chloride density gradient centrifugation, DEAE-cellulose ion exchange chromatography, and Sepharose 4B molecular sieve filtration. The use of selective ethanol precipitation and ion exchange, produced a keratan sulfate proteoglycan fraction and a chondroitin sulfate proteoglycan fraction, but the question of whether the two glycosaminoglycans were present on the same core protein remained unresolved.

The present study was undertaken to define conditions where explants of intact cornea or of cornea stroma from rhesus monkeys could be maintained for a limited period of time in organ culture. The biosynthesis of stroma proteoglycans was followed using [35S]sulfate in combination with either [3H]mannose or [3H]glucosamine. In this way, it was possible to obtain proteoglycans and glycoproteins with sufficient amounts of incorporated radiolabeled from small amounts of tissue to determine suitable methods for isolating, purifying, and characterizing the proteoglycans. Two distinct proteoglycans, one containing chondroitin sulfate and one containing keratan sulfate, were identified. These proteoglycans were subsequently isolated by the same procedures directly from rhesus monkey corneal stroma in sufficient quantities to characterize them chemically. The explant culture system, then, provides an excellent model for characterizing native corneal stroma proteoglycans and for studying their biosynthesis.

EXPERIMENTAL PROCEDURES

Materials—Chondroitinase ABC (Proteus vulgaris), chondroitinase AC (Arthrobacter aurescens), and disaccharide standards were purchased from Miles; [3H]glucosamine-labeled hyaluronic acid was a gift of Dr. James Kimes (18). Papain (9 times crystallized; Sigma) and tissue culture materials (calf serum, medium, etc.) were from Grand Island Biological Co.; [2-3H]mannose (15 Ci/mmol), [6-3H]glucosamine (300 Ci/mmol), and [35S]sulfate (100 Ci/mmol) were purchased from New England Nuclear; Sepharose and Sephadex molecular sieve gels were from Pharmacia; and aqueous counting scintillant (ACS) was from Amersham Corp. Keratan sulfate β endogalactosidase was a gift from Dr. Sakaru Suzuki, Department of Chemistry, Faculty of Science, Nagoya University, Chikusa-Ku, Nagoya, 464, Japan.

Column Chromatography—Columns of Sepharose CL-4B, Sepharose CL-6B, and Sephacryl S-200 (90 × 1.5 cm for analytical; 140 × 1.5 cm for preparative) were eluted with 4 M guanidine HCl, 20 mM Tris-HCl, pH 7, under a 40 cm head. Effluent fractions (3.0 ml) were collected.

Radioisotope Analyses—Aliquots of 0.020 ml for eluent fractions in 4 M guanidine HCl were counted directly in 10 ml of ACS. Larger aliquots were diluted with an equal volume of 70% ethanol before the addition of the ACS. Samples were counted in a Beckman LS 355 scintillation counter and corrections were made for spillover with dual-labeled samples using an external standard method.

Analytical Procedures—Uronic acids were determined by an automated version of the carbazole method (19). Hexosamine was measured by an anthrone reaction (20) and proteins by the Lowry procedure (21). Hexosamines were separated on a Durrum amino acid analyzer and identified by an anthrone reaction (20) and proteins by the Lowry procedure (21). Amino acids were separated on a Durrum amino acid analyzer after hydrolysis of samples under vacuum in 6 N HCl at 110°C for 8 h. Amino acids were determined on the amino acid analyzer after hydrolysis of samples under vacuum in 6 N HCl at 110°C for 8 h. Amino acids were determined on the amino acid analyzer after hydrolysis of samples under vacuum in 6 N HCl at 110°C for 8 h. The proportion of incorporated [3H]mannose which remained in mannose was determined by gas-liquid chromatographic analysis of boronic acid derivatives of the neutral sugars as previously described (22, 23). The samples were...
hydrolyzed in 3 N HCl for 3 h at 100°C before subsequent derivitization steps. Corrections were not made for losses due to hydrolysis.

**Orgon Culture**—Corneas were obtained from freshly enucleated eyes of rhesus monkeys used for separate experiments. The intact corneas were incubated in approximately 20 ml of basal medium essential medium containing 5% fetal calf serum, 100 μg/ml of penicillin, and 50 μg/ml of streptomycin. In some initial experiments, the epithelial and endothelial layers were separated from the stroma by scraping and the stroma was incubated separately. Cultures were maintained for different times, usually 18 h at 37°C, in a humidified incubator with a 5% air, 5% CO₂, atmosphere. Macromolecules were labeled with [³⁵S]sulfate, 50 μCi/ml, used either alone or in combination with either [²-H]mannose, 200 μCi/ml, or [³-¹H]glucosamine, 60 to 100 μCi/ml. Corneas were removed from the medium at the end of the incubation period, rinsed briefly in phosphate-buffered saline, pH 7.4, and stored at -20°C until analyzed. Media fractions were adjusted to 4 M guanidine HCl and the proportions of labeled macromolecules were determined by eluting aliquots on PD-10 Sephadex G-25 columns using 4 M guanidine HCl, 20 mM Tris-HCl, pH 7, as the eluant (24).

**Proteoglycan Extraction and Fractionation**—Each frozen labeled cornea was individually thawed to 4°C, the stroma was rapidly scraped free of epithelium and endothelium and immediately placed in 20 ml of a basal medium containing 0.1 M guanidine HCl, 0.01 M EDTA, 0.01 M sodium acetate, pH 5.8, 0.1 M 6-aminohexanionic acid, and 0.006 M benzamidine-hydrochloride (25). The extraction, 0.5 ml/cornea, was done at 4°C for 24 h. The extract was decanted, the residues were re-extracted for an additional 24 h in the same volume at 4°C, and the two extracts were combined. The proportions of labeled macromolecules remaining in the combined extracts were determined by solubilizing the tissue with papain, 0.5 mg of papain/ml, in 1.0 M sodium acetate, pH 6.5, 0.005 M cysteine, 0.005 M sodium EDTA, 60°C, for 4.5 h. Unincorporated radioisotope was removed from the combined 4 M guanidine HCl extracts and papain digests by chromatography on PD-10 Sephadex G-25 columns as described above. The macromolecular components (the excluded fractions) of the extracts were chromatographed on Sepharose CL-4B in 4 M guanidine HCl containing 20 mM Tris-HCl, pH 7.0. Effluent fractions were monitored for radioactivities, and the tubes containing the peak fractions were pooled as described under "Results and Discussion." Between 87% and 91% of the radioactivity applied to the column was recovered. Portions of the pooled fractions from the column eluents were dialyzed against distilled water and lyophilized to dryness. These samples were solubilized in 2% SDS containing 10 ml sodium phosphate, pH 7.0, reduced with dithiothreitol (10 mM), heated at 37°C for 10 min, and electrophoresed in a slab polyacrylamide gel prepared with a 3% stacking gel and 5% running gel as described previously (25). The extracted and dialyzed fractions were electrophoretically fractionated using an ISCO density gradient fractionator. Densities of the effluent fractions were determined using a 100-μl pipette as a pyknometer, and radioactivities in the fractions were measured as described above.

**Glycosaminoglycan Characterization**—Glycosaminoglycans were isolated from portions of the proteoglycan fractions which were recovered from Sepharose CL-4B eluant, dialyzed against distilled water, and lyophilized. Between 85 and 90% of the radioactivity applied to the column was recovered. Portions were then dissolved in 1 ml of buffer and digested with 0.5 mg of papain as described above. Alternatively, portions were dissolved in 0.05 M NaOH, 1 M NaBH₄, and incubated at 45°C for 48 h (28). Excess borohydride was removed by acidifying the solution with glacial acetic acid. The glycosaminoglycans were then run on Bio-Gel A50M anion-exchange chromatogram and the fractions were analyzed for a number of different parameters. Portions of the glycosaminoglycans were digested with chondroitinase ABC as described above and then chromatographed on Sephadex G-50 superfine (0.5 × 90 cm column) in 0.050 M Tris buffer, pH 7.2. Aliquots were counted as described above and the per cent digestion was determined by calculating the proportion of radioactivity shifting from the Vₒ to lower molecular weight components. A portion of the radioactivity in the Vₒ was then digested with keratan sulfate-beta-endogalactosidase (0.05 M Tris, pH 7.2, 16 h at 37°C) and rechromatographed on G-50 superfine. The shift in radioactivity again determined the per cent digested. Undigested samples served as controls.

**Preparation of Proteoglycans for Biochemical Analyses**—Twenty-five corneas were collected over a period of time from rhesus monkeys being used in separate experiments. Each cornea was rapidly frozen on dry ice immediately after excision and stored at -70°C until used. Each was thawed separately, the stroma was freed of epithelium and endothelium, before extraction, and then extractions were centrifuged at 37,000 rpm for 120 min. TheFractions were determined using a 100-μl pipette as a pyknometer, the effluent was centrifuged in dissociative cesium chloride density gradients at 4°C. This procedure has been shown to precipitate corneal proteoglycans (24). Approximately 90% of the [³⁵S]-labeled proteoglycans were recovered in the pellet. The pellet was redissolved in 4 M guanidine HCl containing 20 mM Tris-HCl, pH 7.0, and the solution was fractionated on a preparative Sepharose CL-4B column. Aliquots from the effluent fractions were counted for radioactivity and the remainder of each was dialyzed extensively against distilled H₂O. The remainder were stored at -70°C until subsequent analyses. Several analyses were done on all or some of these fractions: hexuronic acid by the carbazole procedure (19), neutral sugars by the anthrone procedure (20), hexosamines by the amino acid analyzer, and protein by the Lowry (21) procedure.

**RESULTS AND DISCUSSION**

**Characteristics of Cultures**—Initial experiments were done to determine the kinetics of incorporation of the precursors, [³⁵S]sulfate and [¹H]glucosamine, into macromolecules by intact corneal explants. The majority of the labeled macromolecules were found in the 4 M guanidine HCl extracts of the stroma (Table I). The amounts of labeled macromolecules recovered in media fractions were less than 20% and these fractions were not analyzed further. Removing the epithelium and endothelium from the stroma, before incubation instead of afterwards, did not alter the proteoglycan profiles obtained in the molecular sieve chromatogram analyses described below. The kinetics of incorporation of the precursors into macromolecules was linear and the incorporated [¹H]/[³⁵S] ratios were constant over the 18-h incubation period. Therefore, to obtain maximum incorporated radioactivity, all subsequent analyses and experiments utilized labeled samples derived from 18-h incubations of intact corneal explants with the proteoglycans being extracted from the stroma after removing epithelial and endothelial layers.
Isolation of Proteoglycans—Table II shows that 70, 68, and 52% of the incorporated $^{35}$S activity, $^3$H activity (with $[^3$H]-glucosamine as the precursor), and $^{3}$H activity (with $[2^{-}$H]-mannose as a precursor), respectively, were extracted from labeled stroma by 4 M guanidine HCl after 18-h labeling. Fractionation of these extracts on Sepharose CL-4B yielded four $^3$H-labeled peaks in each case (Fig. 1). The excluded Peak 1 for the experiment utilizing $[^3$H]glucosamine contained only a small amount of the total $^{35}$S activity, but approximately 90% of the $^3$H activity. Glycosaminoglycans derived by papain treatment of the excluded peak were digested by chondroitinase AC and separated by paper chromatography. The results indicated that 12% of the $^3$H activity was in hyaluronic acid. The remaining 88% of the $^3$H activity did not migrate from the origin. Peaks 2 and 3 contained over 85% of the total $^{35}$S activity. The presence of incorporated $^{35}$S label in Peaks 2 and 3 indicated that they contain proteoglycans. Peak 4 contained $^3$H activity with only small amounts of $^{35}$S activity and it was much more pronounced when $^3$H-mannose was used as a precursor. Peak 4, then, probably contains primarily newly labeled glycoproteins. It was not studied further. Both Peaks 2 and 3 were labeled when $^3$H-mannose was used.

Portions of Peaks 2 and 3 from the $[^3$H]mannose experiment and from a separate experiment utilizing only $[^3$H]glucosamine were electrophoresed in 5% polyacrylamide slab gels containing sodium dodecyl sulfate. Fluorographs (Fig. 2) of the gels showed that Peak 2 consisted of a single wide band at a position where a globular protein of $(M_r = \sim 170,000)$ would migrate. This band was present for both the sample incubated with $[^3$H]glucosamine (Fig. 2A) and that labeled with $[^3$H]mannose (Fig. 2C). This band was also labeled when $[^3$S]sulfate was used as a precursor (not shown). Peak 3 from both $^3$H-labeled samples contained several bands migrating in the region of globular proteins of molecular weight from 70,000 to 100,000 (Fig. 2, B and D). Lower molecular weight material was observed at the buffer front. When $^{35}$Sulfate was used, the components in the range of molecular weights from 70 to $100 \times 10^3$ were more intensely labeled than those at the buffer front. Also, Peak 3 material derived from $[^3$H]mannose labeling contained more radioactivity in the front than Peak 3 material derived from $[^3$H]glucosamine labeling. These observations suggest that the low molecular weight components at the buffer front in the Peak 3 samples were contaminating glycoproteins.

Samples from Peaks 2 and 3 that were isolated from corneas labeled with both $[^3$H]mannose and $[^3$S]sulfate were subjected to CsCl gradient centrifugation in 4 M guanidine HCl (Fig. 3). The Peak 2 component exhibited a single, broad band with an average density of 1.34 g/ml that contained both $^3$H and $^{35}$S activity (Fig. 3A). The Peak 3 component exhibited two bands: a lower density component (average density of 1.24 g/ml) which contained mostly $^3$H activity, and a higher density component (average buoyant density of 1.32 g/ml) that contained both $^3$H and $^{35}$S activity (Fig. 3B). The lower buoyant density band corresponds to the lower molecular weight glycoproteins which contain the Peak 3 fraction. Thus, a combination of [Sepharose CL-4B column chromatography followed by density gradient centrifugation provides a better purification for the proteoglycans in Peak 3](http://www.jbc.org/). Tubes 6 to 10 of the density gradient were pooled for both Peaks 2 and 3 and further characterized. Subsequent neutral

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**Table I**

Radioisotope incorporation into intact corneas

Five intact corneas were incubated for 18 h in medium containing both $[^3$H]glucosamine and $[^3$S]sulfate. Each was separated into three tissues and each tissue was extracted in 4 M guanidine HCl. Incorporation was determined by passage through PD-10 columns.

<table>
<thead>
<tr>
<th>Label</th>
<th>Epithelium</th>
<th>Stroma</th>
<th>Endothelium</th>
<th>Media</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^3$H]Glucosamine</td>
<td>2,161 (11%)</td>
<td>1,450 (7%)</td>
<td>3,700 (19%)</td>
<td>19,414</td>
<td></td>
</tr>
<tr>
<td>$[^3$S]Sulfate</td>
<td>325 (4%)</td>
<td>433 (8%)</td>
<td>1,100 (14%)</td>
<td>7,708</td>
<td></td>
</tr>
</tbody>
</table>

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**Table II**

Extractability of incorporated radiolabel from cornea stroma

Three corneas in each experiment were labeled for 18 h in media containing either $[^3$H]glucosamine, $[^3$S]sulfate, or $[^3$H]mannose. The epithelium and endothelium layers were then removed and the stroma extracted in 4 M guanidine-HCl. The residue was digested with papain and incorporation into the residue and extract was determined by passage on PD-10 columns.

<table>
<thead>
<tr>
<th>Extract</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[^3$H]Glucosamine</td>
<td>68</td>
</tr>
<tr>
<td>$[^3$S]Sulfate</td>
<td>70</td>
</tr>
<tr>
<td>$[^3$H]Mannose</td>
<td>52</td>
</tr>
</tbody>
</table>

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* $[^3$H]Glucosamine as precursor.
* $[^3$S]Sulfate as precursor.
* Per cent of total incorporated radioactivity.
Proteoglycans of Primate Cornea

MW x 10^3
-220
-155
-94
-68

FIG. 2. Fluoroautoradiogram of 5% polyacrylamide gels after electrophoresis of Peaks 2 and 3. A, peak 2, [3H]glucosamine label used as precursor; B, Peak 3, [3H]glucosamine used as precursor; C, [3H]mannose-labeled Peak 2; and D, [3H]mannose-labeled Peak 3. Molecular weight markers denote migration position of globular proteins. Arrow indicates position of buffer front.

FIG. 3. Dissociative CsCl density gradient profiles of Peaks 2 and 3 labeled with [3H]mannose and [35S]sulfate. A, Peak 2; B, Peak 3.

Aliquots of the papain digests of Peak 2 from the [35S]-sulfate and [3H]glucosamine sample were also chromatographed on Sepharose CL-6B (Fig. 5). The intact proteoglycan eluted as a single peak with K_d = 0.20 (Fig. 5A). Digestion of the protein portion of the proteoglycan with papain (Fig. 5B) produced a major peak with K_d = 0.30 which contained both 35S and 3H activity and the second, 3H-labeled oligosaccharide peak eluting near the total column volume. The elution profiles were nearly the same when the Peak 2 proteoglycans were treated with the alkaline-borohydride procedure (Fig. 5C).

The glycosaminoglycan peak from the papain digest of Peak 2 material was recovered from the excluded volume of the S-200 elution (Fig. 4A), and aliquots were digested with chondroitinase AC or with chondroitinase ABC. Chondroitinase ABC digested 88% of the labeled material as shown by paper chromatography. The disaccharides consisted of 27%, 4-S, 15%, 6-S, and 47%, chondroitin (Table III). Chondroitinase AC (30) digested only 60% of the 3H activity indicating the presence of some iduronic acid in the glycosaminoglycan chains (5). The data indicate that the Peak 2 proteoglycan contains chondroitin-dermatan sulfate copolymer. The K_d of the free chains on Sepharose CL-6B corresponds to a M_r = ~55,000 (31). The K_d for the intact proteoglycan would correspond to a single chondroitin sulfate chain of M_r = ~90,000.

Papain digests of aliquots of the Peak 3 proteoglycan (Fig. 1) were chromatographed on Sephacryl S-200 (Fig. 6). For the sugar analyses of these samples by gas-liquid chromatographic and radioisotopic counting procedures showed that greater than 96% of the total 3H radioactivity present was contained in mannose. This indicates that both proteoglycan fractions contain mannose residues and probably, therefore, contain oligosaccharides of the glycoprotein type.

Glycosaminoglycans in the Proteoglycans—Aliquots of the Peak 2 fraction from the samples in Fig. 1 were digested with papain and fractionated on Sephacryl S-200 (Fig. 4). For the sample labeled with both [35S]sulfate and [3H]glucosamine, a major peak eluted at the void volume that contained both 35S and 3H activity and a minor peak eluted near the total column volume that contained predominantly 3H activity. From the calibration of the Sephacryl S-200 column with oligomers of known molecular weight derived from hyaluronic acid, the molecular weight of this smaller component was estimated to be about 2,500. For the sample labeled with [3H]mannose, the small molecular weight component was essentially the only one observed. This component, then, probably consists of glycoprotein oligosaccharides.

Fig. 4. Sephacryl S-200 chromatography of papain digests of Peak 2. A, [3H]glucosamine and [35S]sulfate used as precursors; B, [3H]mannose used as precursor. Arrows denote elution position of oligosaccharide standards, of known molecular weight, derived from hyaluronic acid.
Proteoglycans of Primate Corneal Stroma

Fig. 5. Sepharose CL-6B chromatography of Peak 2, ["H]-glucosamine and ["35S]sulfate used as precursors. A, intact proteoglycan (Peak 2); B, papain digest of Peak 2; and C, sodium hydroxide-sodium borohydride digest of Peak 2.

Table III
Disaccharides generated by chondroitinase ABC digestion of glycosaminoglycans

Glycosaminoglycans were digested with chondroitinase ABC and the products were separated by paper chromatography.

<table>
<thead>
<tr>
<th></th>
<th>Peak 2 glycosaminoglycans</th>
<th>Peak 3 glycosaminoglycans</th>
<th>% total [&quot;H&quot; activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chondroitinase-resistant</td>
<td>12</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Chondroitin 6 SO₄</td>
<td>15</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Chondroitin 4 SO₄</td>
<td>27</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>Chondroitin</td>
<td>46</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Hyaluronate</td>
<td>&lt;1</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

* ["H]Glucosamine as precursor.

sample in which ["35S]sulfate and ["H]glucosamine were used as precursors, the majority of the ["35S] label eluted as a broad peak which co-eluted with a portion of the ["H] label (Fig. 6A). A proportion of the ["H] activity eluted later in the oligosaccharide region. For the Peak 3 sample labeled with ["H]-mannos, most of the activity was found in the later eluting fraction (Fig. 6B); only about 15% eluted in the glycosaminoglycan region. Since Peak 3 contained some glycoprotein contaminants, the experiment was repeated for a Peak 3 sample in which ["35S]sulfate and ["H]mannos were used as precursors that was subsequently purified by a CsCl density gradient as indicated in Fig. 3B above. The elution profile of a papain digest of this sample on S-200 also contained the later eluting, ["H]-labeled peak indicative of the presence of glycoprotein-type oligosaccharides in this proteoglycan (Fig. 7). Compared to the elution position of known standards, the broad, ["35S]- and ["H]-labeled glycosaminoglycan peak indicated in Fig. 6A exhibited an average molecular weight of approximately 7,000. Only 10% of this glycosaminoglycan fraction was digested by chondroitinase ABC treatment (Table III).

A peak 3 proteoglycan fraction (in which ["35S]sulfate and ["H]glucosamine were used as precursors) was chromatographed on Sepharose CL-6B before and after treatment with papain or alkaline-borohydride (Fig. 8). The intact proteoglycan eluted with Kᵦ = 0.3 (Fig. 8A), whereas treatment with either papain or alkaline-borohydride shifted the elution position of the ["35S]-labeled material to a Kᵦ of 0.55. Based on this Kᵦ, the average molecular weight of the glycosaminoglycans can be estimated to be about 15,000, provided they have similar elution profiles to that of chondroitin sulfate and the

Fig. 6. Sephacryl S-200 chromatography of papain digests of Peak 3. A, ["H]glucosamine and ["35S]sulfate were used as precursors; B, ["H]mannos used as precursor. Arrows denote elution position of oligosaccharide standards, of known molecular weight, derived from hyaluronic acid.
Proteoglycans of Primate Corneal Stroma

FIG. 7. Sephacryl S-200 chromatography of papain digests of CsCl density gradient purified Peak 3. Tubes 6 to 10 in Fig. 3B were pooled, digested with papain, and chromatographed. [3H]Man- nose and [35S]sulfate were used as precursors.

FIG. 8. Sepharose CL-6B chromatography of Peak 3, [3H]-glucosamine and [35S]sulfate used as precursors. A, intact proteoglycan (Peak 3); B, papain digest of Peak 3; and C, sodium hydroxide-sodium borohydride digest of Peak 3.

treatments release single chains (31). This is twice the molecular weight value estimated on Sephacryl S-200 where oligosaccharides derived from hyaluronate were used as standards, Fig. 6, above. The Kd of the intact proteoglycan would correspond to a glycosaminoglycan, M = 55,000.

The average size of the keratan sulfate chains released by the alkaline-borohydride treatment was the same as that released by papain digestion. The linkage between keratan sulfate and protein in corneal proteoglycans involves an N-glycoside bond between glucosamine and asparagine (18, 32, 33) and, therefore, should not be alkali-labile. The results from the alkaline-borohydride treatment, then, suggest that the polypeptide around the linkage regions for the keratan sulfate chains are extensively hydrolyzed. Similarly, the polypeptide regions to which the later eluting, [3H]-labeled oligosaccharides are attached must be susceptible to the alkaline-borohydride treatment if they are linked by N-glycoside bonds.

The glycosaminoglycans from Peaks 2 and 3 proteoglycans (in which [35S]sulfate and [3H]glucosamine were used as precursors) were also sequentially digested with chondroitinase ABC followed by keratan sulfate-β-endogalactosidase as described under "Experimental Procedures." Ninety percent of the glycosaminoglycans from Peak 2 were sensitive to chondroitinase ABC while 74% of the glycosaminoglycans from Peak 3 were sensitive to keratan sulfate-β-endogalactosidase (Table IV).

Glycosaminoglycans in the Residue—The residual material not extracted from the stroma by 4 M guanidine HCl was digested with papain and fractionated on Sephacryl S-200 (Fig. 9). Most of the [35S] and [3H] activity for the residue from tissue labeled with [35S]sulfate and [3H]glucosamine eluted in the void volume (Fig. 9A) with a smaller peak which contained primarily [3H] activity eluting later. For the tissue labeled with [3H]mannose, this later eluting peak was the predominant component, Fig. 9B. A broad zone containing primarily [35S] activity appeared between the two peaks indicated on Fig. 9A. Chondroitinase ABC digested 93% and chondroitinase AC digested 64% of the [35S] activity in the void volume peak, whereas, these enzymes digested only 5% of the [35S] activity between the two peaks. The excluded component, then, represents primarily the chondroitin-dermatan sulfate, whereas the included component probably represents keratan sulfate. The labeled glycosaminoglycans remaining in the residue, then, appear to be very similar to those found in the purified proteoglycans in the extracts.

Proteoglycans Directly Isolated From Corneal Stroma—The results of the labeling experiments indicate that the stromocytes in corneal stroma of rhesus monkeys synthesize two proteoglycan molecules, one with chondroitin-dermatan sulfate and one with keratan sulfate. The following experiments indicate that these two proteoglycans are present in stroma in vivo. Stromal tissue from 25 corneas was extracted with 4 M guanidine HCl. Labeled material from tissue incubated with [35S]sulfate and [3H]glucosamine was added, and the proteoglycan fractions were isolated as described under

TABLE IV

| Sensitivity of 35S-labeled glycosaminoglycans to chondroitinase ABC and keratanase |
|---------------------------------|-----------------|
| Peak 2 glycosaminoglycans      | Peak 3 glycosaminoglycans |
| Chondroitinase ABC             | 90              | 7               |
| Keratanase                     | 6               | 75              |
| Resistant                      | 3               | 17              |

* Keratan sulfate-β-endogalactosidase.
**Proteoglycans of Primate Corneal Stroma**

**Experimental Procedures.** The elution profiles of the labeled molecules in the effluent fractions are shown in Fig. 10C. The ethanol precipitation step selectively precipitated the Peak 2 and Peak 3 molecules. (Contrast Fig. 10C with Fig. 1A.) The major hexosamine peak eluted in the position of the Peak 2 proteoglycan, whereas the major hexose peak eluted in the position of the Peak 3 proteoglycan (Fig. 10, A and C). The major protein peak eluted slightly after the Peak 3 proteoglycan (Fig. 10B). Hexosamine analyses of fractions through these two peaks are given in Table V. Galactosamine was predominant in the Peak 2 component while glucosamine was predominant in Peak 3. These chemical analyses are consistent with the presence of chondroitin-dermatan sulfate in the Peak 2 proteoglycan and keratan sulfate in the Peak 3 proteoglycan.

The peak fractions for the chondroitin-dermatan sulfate and keratan sulfate proteoglycans (51 and 56, respectively) were further purified by dissociative CsCl density gradient centrifugation. The proteoglycans banding between 1.280 and 1.400 (see Fig. 3) were isolated and analyzed for amino acid and hexosamine contents (Table VI). Both proteoglycans were rich in aspartic acid, glutamic acid, and leucine. The chondroitin-dermatan sulfate proteoglycan contained substantially more glycine and cysteine and substantially less methionine and tyrosine than the keratan sulfate proteoglycan. The amino acid composition of both proteoglycans differed significantly from those of cartilage proteoglycans (34-36). By calculating the disaccharide content from the hexosamine content, it was possible to estimate the percentage of protein in each of these proteoglycans. The chondroitin-dermatan sulfate proteoglyc-
Table VI

<table>
<thead>
<tr>
<th>Amino acid and hexosamine composition of proteoglycans in Tubes 51 and 56 after purification by CsCl density gradient centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residues of amino acids/1000 No. 51 No. 56</td>
</tr>
<tr>
<td>---------------------------------------------------------</td>
</tr>
<tr>
<td>Aspartic acid</td>
</tr>
<tr>
<td>Threonine</td>
</tr>
<tr>
<td>Serine</td>
</tr>
<tr>
<td>Glutamic acid</td>
</tr>
<tr>
<td>Proline</td>
</tr>
<tr>
<td>Glycine</td>
</tr>
<tr>
<td>Alanine</td>
</tr>
<tr>
<td>Cysteine</td>
</tr>
<tr>
<td>Valine</td>
</tr>
<tr>
<td>Methionine</td>
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<tr>
<td>Isoleucine</td>
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<tr>
<td>Leucine</td>
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<tr>
<td>Tyrosine</td>
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<tr>
<td>Phenylalanine</td>
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<tr>
<td>Histidine</td>
</tr>
<tr>
<td>Lysine</td>
</tr>
<tr>
<td>Arginine</td>
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<tr>
<td>Glucosamine residues/1000 amino acids</td>
</tr>
<tr>
<td>Galactosamine residues/1000 amino acids</td>
</tr>
</tbody>
</table>

This study demonstrates that there are two distinct proteoglycans present in intact rhesus monkey corneal stroma and synthesized by corneal explants in culture. The larger is a chondroitin-dermatan sulfate proteoglycan as shown by its hexuronic acid content, high galactosamine content, and the sensitivity of the glycosaminoglycan chains to chondroitinase ABC. The smaller is a keratan sulfate proteoglycan as shown by its hexose content, high glucosamine content, the resistance of the glycosaminoglycan side chains to chondroitinase ABC, and its sensitivity to keratan sulfate-β-endogalactosidase.

**General Discussion**

The results of this study are in general agreement with the data obtained by Axelson and Heinegard (37) for proteoglycans of the bovine corneal stroma, particularly with reference to the possible presence of glycoprotein-type oligosaccharides as part of the proteoglycans. There were, however, some differences. First, Axelson and Heinegard were not able to resolve the two proteoglycans on Sepharose 4B. In the present study, Sepharose CL-4B chromatography was done in dissociative conditions (4 M guanidine HCl) while Axelson and Heinegard used associative conditions. This may suggest some interaction between the two proteoglycans under associative conditions. Alternatively, the hydrodynamic volumes of the two proteoglycans may be similar under associative conditions but different under dissociative conditions. Further, the amino acid compositions of the two bovine stroma proteoglycans differed from our analyses of monkey stroma proteoglycans. Their material was obtained by DEAE-chromatography and ethanol precipitation while ours was obtained by ethanol precipitation, molecular sieve filtration, and CsCl density gradient centrifugation.

The proportions of the two proteoglycans present in the stroma differed from the proportions of the two proteoglycans synthesized by the stromacytes in the stroma (compare Figs. 1A and 1C with Table V). The decreased synthesis of the keratan sulfate proteoglycan relative to the chondroitin sulfate proteoglycan may represent a partial loss of the capacity to synthesize this proteoglycan since other studies have shown that stromacytes in cell culture fail to make keratan sulfate glycosaminoglycans (38).

The data above indicate that the corneal stroma proteoglycans differ greatly from cartilage proteoglycans. Cartilage proteoglycans contain protein cores which have both chondroitin sulfate and keratan sulfate side chains. There are an average of approximately 100 chondroitin sulfate side chains and 50 keratan sulfate side chains per protein core yielding an average molecular weight of 2 x 10^6. Only about 10% of the structure is protein. The corneal proteoglycan is probably significantly less. Digestion of the sylated proteins in SDS gels, the true molecular weight of this proteoglycan was estimated from the electrophoretic mobility of proteins of known molecular weight, the molecular weight of the proteoglycan. Biochemical analyses indicated that the chondroitin-dermatan sulfate proteoglycan consists of a protein core with only one, or at most two, chondroitin-dermatan sulfate side chains and with several glycoprotein-type oligosaccharide side chains.
stromal proteoglycans are considerably smaller than the cartilage monomer and are approximately 70% protein. The chondroitin sulfate and keratan sulfate side chains of corneal proteoglycans are bound to different protein cores to produce two separate proteoglycans. Although the stromal proteoglycans are smaller, their keratan sulfate side chains are the same size as the cartilage keratan sulfate side chains, while their chondroitin sulfate side chains are nearly twice as large as the cartilage chondroitin sulfate side chains (39).

These studies demonstrate the usefulness of combining labeling of proteoglycans in explant cultures with biochemical analyses. The proteoglycans synthesized by the cells in the explants are similar to, if not identical with, their counterparts in the tissues, although they are synthesized in different proportions than they accumulate. Therefore, the explant system will serve as an important model system for determining the structure of corneal proteoglycan as well as for identifying possible alterations which may occur in proteoglycans in certain corneal diseases or during embryonic corneal development.

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